Data Requirement:	EPA DP Barcode	406079
	OECD Data Point	231
	EPA MRID	48675901
	EPA Guideline	890.1100
		Amphibian Metamorphosis Assay (Frog)

Test Material: Bifenthrin Technical **Purity (%):** 93.6

Common Name Bifenthrin

Chemical Name IUPAC 2-Methyl-3-phenylphenyl)methyl (1S,3S)-3-[(Z)-2-chloro-3,3,3-trifluoroprop-1-enyl]-2,2-dimethylcyclopropane-1-carboxylate

CAS Name Not reported

CAS No. 82657-04-3

Synonyms None reported

EPA PC Code 128825

Primary Reviewer: Elizabeth Krupka **Signature:**

Environmental Scientist, CDM Smith **Date:** 6/27/2013

Secondary Reviewer: Teri S. Myers **Signature:**

Environmental Scientist, CDM Smith **Date:** 7/22/2013

Primary Reviewer: [.....] **Date:** [.....]

[EPA/OECD/PMRA]

Secondary Reviewer(s): [.....] **Date:** [.....]

[EPA/OECD/PMRA]

Date Evaluation Completed: [dd-mmm-yyyy]

CITATION: Schneider, S.Z., T. Z. Kendall, and H.O. Krueger. 2012. Bifenthrin: Amphibian Metamorphosis Assay for the Detection of Thyroid Active Substances. Unpublished study performed by Wildlife International, Ltd., Easton, Maryland 21601. Laboratory project number 701A-106. Study sponsored by Consumer Specialty Products Association for the Bifenthrin Task Force Steering Committee/Joint Venture. Study completed August 14, 2012.

[Instructions, prompts, and example values for the individual(s) completing the DER are shown in the DER template in bracketed red text; these instructions and examples do not need to remain visible in the completed DER.]

Guideline recommendations are provided in italics; these recommendations should remain visible in the completed DER.

Disclaimer: *The guideline recommendations in this DER template are offered as a general reference to aid in preparation of the DER. The purpose of these recommendations is not to serve as substitute for the Test Guidelines, nor to provide any guidance on how the study should be conducted.*

EXECUTIVE SUMMARY

The 21-day assay of Bifenthrin on amphibian metamorphosis of African clawed frog (*Xenopus laevis*) was studied under flow-through conditions. Amphibian larvae (80 per control and treatment group, NF stage 51) were exposed to a negative control, solvent control, and nominal concentrations of 0.000030, 0.00010, and 0.00030 mg a.i./L; the %CV of measured concentrations exceeded 20% over the course of the study (i.e., 22-55%). The time-weighted average (TWA) concentrations (calculated by the reviewer) were <0.0000150 (<LOQ; control), 0.000012, 0.000051, and 0.00023 mg a.i./L The test system was maintained at 21.9 to 22.7°C and a pH of 7.9 to 8.1.

Bifenthrin significantly delayed median Nieuwkoop-Faber (NF) developmental stage at 21 days in the 0.00023 mg a.i./L treatment group (median NF=57) relative to the negative control (median NF=58; $p<0.05$, Dunnett's test). Effects on thyroid gland histopathology were observed at all treatment levels. Mild gland atrophy was notable in the high treatment group relative to the negative control group. Clinical signs (*i.e.*, behavioral and other sublethal effects) including tail curvature and small size were observed in all treatment groups, with no apparent pattern. Tail curvature was seen in 53% (32/60) of tadpoles in the negative control and in 80% (48/60), 60% (36/60), and 63% (38/60) tadpoles the 0.000012, 0.000051, and 0.00023 mg a.i./L treatment levels, respectively. The study author reported that in feeding trials it had been shown that feeding rates contributed to the amount of curvature shown; therefore tail curvature was believed to be related to diet and not thyroid-related. Small size was seen in one tadpole in the negative control and in all treatment groups except the 0.000051 mg a.i./L treatment group, where three tadpoles were observed to exhibit small size. Asynchronous development was observed in one tadpole in the 0.000051 mg a.i./L treatment group. Late stage development (NF=61-63) was observed in 3, 3, 2, 4 and 2 tadpoles in both control groups and at the 0.000012, 0.000051, and 0.00023 mg a.i./L treatment levels, respectively; late stage tadpoles were excluded from analyses of growth. In terms of growth effects, bifenthrin significantly promoted 7-day hind-limb length (HLL), snout-to-vent length (SVL), and body weight at 0.000051 mg a.i./L, relative to the negative control ($p<0.05$, Dunnett's). On Day 21, SVL was significantly greater than the negative control at the 0.000012 mg a.i./L level and wet weight was significantly promoted at all treated levels, relative to the negative control ($p<0.05$, Dunnett's).

The reviewer cautions interpretation of the results of this study. The reviewer's analysis detected significant

differences between the negative and solvent control groups for most endpoints ($p<0.05$), with a consistent pattern of promotion in the solvent control, relative to the negative control. As a result, the reviewer suspects that solvent interference may have been a factor in this study and could have masked potential negative effects of the test material (which was the directional pattern observed by most endpoints at the high dose, relative to the negative control). Additionally, there was unacceptably high mortality (i.e., 45%) in one replicate of the negative control group, compromising the sample size and statistical power with which to compare this reference to the treated conditions for Day 21 endpoints.

This assay [does or does not] satisfy the Test Order requirement for an Amphibian Metamorphosis Assay (OCSPP Guideline 890.1100).

Results Synopsis:

Test organism NF stage at test initiation: 51

Test organism total length at test initiation (optional): Not reported

Test type: flow-through

Table 1: Summary of Developmental and Thyroid Pathology/Histopathology Effects[1,2] in the Amphibian Metamorphosis Assay (AMA) with Bifenthrin.

Treatment (mg a.i../L) [TWA-measured]	NF Developmental Stage		Hind Limb Length[3]		Asynchronous Development		Thyroid Gross and Histopathology
	Day 7	Day 21	Day 7	Day 21	Day 7	Day 21	Day 21
0.000012	No	No	No	No	No	No	No
0.000051	No	No	No	No	No	Yes	No
0.00023	No	Yes	No	No	No	No	Yes

Abbreviations: Diff. Difference. NA Not applicable.

[1] A "yes" indicates a significant difference based on comparison to the negative (clean water) control, unless otherwise specified.

[2] The criteria for significance are described in the Reviewer's Analysis and Statistical Verification sections of the DER. Conclusions regarding histopathology may be heavily weighted by the expert opinion of a board-certified pathologist.

[3] Hind-limb length is normalized to snout-vent length (SVL).

I. MATERIALS AND METHODS

Guideline Followed: This study was conducted following guidelines outlined in U.S. EPA, Endocrine Disruptor Screening Program Test Guidelines, OPPTS 890.1100, Amphibian Metamorphosis Assay (Frog) (October 2009). The following deviation(s) were noted:

1. The photoperiod (16 hrs light:8 hrs dark) was not consistent with the recommended photoperiod (12 hrs light: 12 hours dark).

2. The water hardness during the definitive exposure period (136-148 mg/L as $CaCO_3$) exceeded the recommended range (40-48 mg/L as $CaCO_3$).

3. The flow-through rate (*ca.* 48.6 mL/min) exceeded the recommended rate (25 mL/min).

4. The feeding rates for tadpoles during the definitive study increased from 15 mg/larvae/day at test initiation to 40 mg/larvae/day by the last week of the test, which was not consistent with the recommended feeding rate of 30 mg/larva/day at test initiation to 80 mg/larva/day in the last week of the test.

5. Mortality in the negative control replicate A (45%) and consequently, the negative control group as a whole

(20%), exceeded the acceptable mortality rate (<10%).

6. The %CV of measured concentrations exceeded 20% for all test material levels over the study duration.

7. The reviewer's analysis detected significant differences between the negative and solvent control groups for most endpoints, with consistent promotion of the solvent control group ($p<0.05$).

Compliance: Signed and dated No Data Confidentiality, GLP, and Quality Assurance statements were provided. This study was conducted in compliance with all pertinent OECD (OECD, 1998) and U.S. EPA (40 CFR, Part 160) Good Laboratory Practice regulations with the following exceptions: periodic analyses of water for potential contaminants were performed using a certified laboratory and standard USEPA analytical methods; preliminary analyses of water iodide concentrations were not performed according to BLP standards; and the characterization and stability of the test substance under the storage conditions of the test site was not determined in accordance with Good Laboratory Practice Standards.

A. Test Material Bifenthrin Technical. 82657-04-3

Description: Liquid

OECD recommends describing water solubility, melting/boiling point stability in water and light, pKa, Pow or Kow, vapor pressure of test compound, expiration date.

Lot No./Batch No. : PL09—251 (Lot No.)

Purity: 93.6%

Impurities: None listed

Stability of Compound: Stable. Mean-measured concentrations yielded recoveries of 70 to 119% of nominal with coefficients of variation of 22.47 to 54.88%.

Storage Conditions of Test Chemicals: Stored under ambient conditions

B. Test Organism

Table 2: General Information About the Test Species and Parental Care.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Species common name:	African clawed frog		*EPA recommends African clawed frog (Xenopus laevis). Western [Africa] clawed frog Silurana (Xenopus) tropicalis may be used as an alternate species[1]; however, a list of all of the necessary protocol deviations to accommodate this species is recommended for inclusion in the study report. The guideline recommends that the performance criteria used to support the reliability of the test be identified.*
Species scientific name:	*Xenopus laevis*		
Species strain (if stated):	Not reported		
Were parents maintained as	Yes	Cultures were maintained for 14 days prior	*EPA recommends that larvae used in the*

[1] U.S. Environmental Protection Agency (EPA). (2011). Corrections and Clarifications on Technical Aspects of the Test Guidelines for the Endocrine Disruptor Screening Program Tier 1 Assays (OCSPP Test Guideline Series 890). March 3, 2011. Office of Chemical Safety and Pollution Prevention (OCSPP), Washington, D.C. (http://www.epa.gov/endo/pubs/assayvalidation/clarificationdoc.pdf).

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
in-house stock?		to test initiation by Wildlife International Ltd. after being obtained from Xenopus I of Dexter, Wisconsin.	*assay be derived from in-house adults.*
Were parental acclimation conditions same as definitive test?	Yes		
Acclimation period for parental frogs (if applicable):	14 days		
Details on parental feeding:	Adult frogs were fed at a rate sufficient to maintain the health of the culture.	Food source is Zeigler Bros., Inc.	
Details on parental health:	Parental frogs were in good health, showing no signs of disease or stress.		

Table 3: Larval Selection and Care.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Best single spawn?	Yes		*EPA and OECD recommend that the best 2 – 3 individual spawns, with a minimum of 1500 larvae/spawn, be evaluated to identify the best single spawn, and that the larvae selected for testing originate from the best single spawn (i.e., the spawns are not co-mixed)*
Number of spawns evaluated (if applicable):	1		
Number of eggs sampled per spawn:	250 eggs from a single spawn were evaluated for viability.	Embryonic viability was checked daily.	
NF stage at test initiation	51		*EPA recommends that the definitive study be initiated with larvae at Nieuwkoop – Faber (NF) developmental stage 51 (≤17 days post-fertilization).*
Age at test initiation:	15 days post-fertilization (dpf)		
Mean total length at test initiation (if reported):	N/A		
Range of total length at test initiation (if reported):	N/A		

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Was the optional size selection method used?	No		
Details on larval selection:	As larvae reached NF stage 51, tadpoles that met the criteria for hind limb morphology were placed one or two at a time into transfer vessels until each vessel contained 20 tadpoles.		
Loading rate (rearing density):	4 larvae/L		*EPA recommends that rearing density (loading rate) not exceed approximately 10 larvae/L culturing system for flow-through systems or 4 tadpoles/L in static-renewal exposure systems.*
Type of food:	Sera Micron		*EPA recommends Sera Micron® throughout pre-exposure (after NF stage 45/46) and*
Source of food:	Sera Micron® USA,		

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
	Montgomeryville, PA		*during the entire 21-d definitive study. If another diet is used, the study report should provide analysis of iodide content and potential contaminants, and the diet should demonstrate equal performance to Sera Micron®.*
Iodide concentration in diet (if known):	Not reported		
Frequency of feeding:	Three times per day		*EPA recommends that feeding occur at least twice per day.*
Details on feeding regime:	The feeding regime was 15 mg/larva/day and increased to 40 mg/larva/day by the end of the test.		*It is recommended that food rations during the pre-exposure period be increased along with larval growth to approximately 30 mg/larva/day by test initiation. EPA and OECD recommend that food rations increase from 30 mg/larva/day at test initiation (Study Day 0-4) to 80 mg/larva/day in the last week of the test (Study Day 15-21).*

C. Exposure System

Table 4: Summary of Information on the Exposure System and Test Vessel Characteristics.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Type of exposure:	Flow-through		*EPA recommends the use of a flow-through system.*
Type of flow-through dilution system (if applicable):	Continuous flow diluter		*Intermittent flow proportional diluters or continuous flow serial diluters are recommended.*[2]
Flow-through rate (if applicable):	*ca.* 48.6 mL/min, based on a 7L fill volume and 10 volume additions per day	The estimated flow rate provided complete volume replacement approximately every 2.9 hours.	*Recommended flow-through rate is 25 mL/min (complete volume replacement ca. every 2.7 hrs).*
Details on toxicant mixing for flow-through systems (if applicable):	A syringe pump was used to deliver volumes of test substance stock solutions and dimethylformanide for the solvent control to the		*Recommended toxicant mixing for flow-through systems: 1) Mixing chamber is recommended but not required; 2) Aeration is not recommended for mixing;3) A demonstration that the test solution is*

[2] Additional guidance for aquatic test design is located in OCSPP Guideline 850.1000, Special Considerations for Conducting Aquatic Laboratory Studies.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
	mixing chambers. The proportion of test solution pumped into each replicate test chamber was checked weekly to ensure flow rates varied by no more than ± the mean flow rate for the replicates. The system was calibrated prior to test initiation.		*completely mixed before introduced into the test system is recommended; 4) The recommended flow splitting accuracy is within 10%.*
Renewal period for static renewal (if applicable):	N/A		*If static renewal is used, EPA recommends 24-hr renewal; renewal period is recommended not to exceed 72 hours.*
Aeration?	No		*EPA recommends maintaining dissolved oxygen concentrations ≥40% air saturation (≥3.5 mg/L). Aeration may be maintained through bubblers. It is recommended to set*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
			bubblers at levels that do not cause stress on the tadpoles.
Source of dilution water:	Well water (40 meter deep well)	Dilution water was prepared by passing the well water through a sand filter to remove particles greater than 25 µm and then pumping it into a storage tank where it was aerated. The water was filtered to 0.45 µm to remove fine particles and was passed through an ultraviolet sterilizer	*EPA recommends natural or reconstituted water; it is recommended that natural water be sterilized with UV and tested for pesticides, heavy metals, and other possible contaminants, including known substrates of the iodine transporter of the thyroid gland (e.g., fluoride, chlorate, perchlorate). OECD accepts any water in which the test species show control survival at least as good as indicated in the test guideline.*
Was dilution water analyzed for pesticides, heavy metals, and other contaminants?	Yes	Dilution water was analyzed for specific conductance, hardness, alkalinity, pH, total organic carbon, pesticides, organics and metals.	
Iodide supplementation in	No. Iodide concentrations		*If reconstituted water is used or if*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
water?	were *ca*. 3-6 µg/L.		*background levels of iodide in natural water are less than 0.5 µg/L, iodide supplementation is recommended. This supplementation is in addition to the recommended dietary source of iodide (e.g.. in Sera Micron).*
Test vessel type/materials:	Test chambers were glass aquaria.		*EPA and OECD recommend that water-contact portions of the system not compromise the study (e.g., all glass vessels or glass vessels with stainless steel frames are acceptable examples).*
Test vessel size:	Specific dimensions were not reported. The total volume was 9 L.		
Fill volume:	*ca.* 7 L		
Additional details on exposure system:	Exposure was conducted in a		

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
	temperature-controlled environmental chamber to maintain the test solution temperatures at 22 ± 1°C.		

Table 5: Summary of Water Quality Characteristics in the Test System.

Parameter	Minimum	Maximum	Mean	Measurement Interval	Guideline Recommendations
Hardness (mg/L as $CaCO_3$)	136	148	143	Weekly	*EPA recommends hardness 40 to 48 mg/L as $CaCO_3$.*
pH	7.9	8.1	8.0	Weekly	*EPA recommends pH 7.5 ± 1, inter-replicate and inter-treatment differentials should not exceed 0.5.*
Dissolved oxygen (mg/L)	4.9	8.4	6.6	Weekly	*EPA recommends dissolved oxygen (DO) >3.5 mg/L (>40% air saturation). OECD recommends DO concentration >3.5 mg/L*

					(>40% air saturation).
Temperature	21.9	22.7	22.2	Temperature was continuously and recorded weekly.	*EPA recommends temperature 22±1°C; inter-replicate and inter-treatment differentials should not exceed 0.5°C.*
Iodide	Not Reported				*EPA recommends aquatic iodide range 0.5 – 10 µg/L (supplemental iodide should not exceed 2 µg/L).*
Ammonia	Not Reported				*General recommendations for frequency of measurements: EPA recommends that water quality parameters be measured in a control and at one test item concentration at least weekly. In static renewal systems, water quality parameters, including ammonia, should be measured just prior to renewal. In addition, EPA recommends that DO be measured at each concentration at least weekly and that temperature be measured continuously. OECD recommends that DO and temperature be*
Fluoride	--	--	0.85	Measured on December 28, 2011.	
Perchlorate	Not Reported				
Chlorate	Not Reported				
Total Alkalinity (mg/L as $CaCO_3$)	176	182	178	Weekly in the negative control and highest treatment group.	
Specific Conductance (µmhos/cm)	379	385	382		

					measured at least weekly and that pH and hardness be measured at least at the beginning and end of the test.

D. Study Design and Additional Experimental Conditions

Table 6: Range-Finding Study Conditions (if Applicable).

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Was a range-finder conducted?	Yes		
If yes, what was the method for determining the highest test concentration in the range-finder?	The high concentration was based on the solubility of the test substance and the reported LC_{50} (0.0000038 – 0.0178 mg/L).		*EPA recommends that the highest test concentration is either the solubility limit of the test compound, 100 mg/L, or demonstrates adequate evidence of toxicity (e.g., ≤10% mortality), whichever concentration is lowest.*

Species:	*Xenopus laevis*		
Life stage:	51		
Test duration:	14 days		
Additional details:	A range-finding study was conducted at nominal concentrations of 0.000016, 0.000054, 0.00018, 0.00060 and 0.00020 mg a.i./L for 14 days. Signs of toxicity observed in the 0.00060 and 0.00020 mg a.i./L treatment groups included surfacing, lethargy, loss of equilibrium, lying on the bottom of the test		

	chamber, and appearing small in comparison to the controls. The high concentration for the definitive test of 0.00030 mg a.i./L was based on the maximum tolerated dose.		

Table 7: Definitive Study Conditions.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Test duration:	21 days		*EPA recommends that the duration of the definitive test be 21 days.*
Method for selecting the highest test concentration in the definitive test:	Range-finder		*EPA recommends that the highest test concentration is either the solubility limit of the test compound, 100 mg/L, or demonstrates*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
			adequate evidence of toxicity (e.g., ≤10% mortality), whichever concentration is lowest.
Reference study citation (if applicable):	N/A		
Separation of test concentrations:	0.33		*EPA recommends that the maximum concentration separation be 0.1 and the minimum be 0.33.*
Number of test concentrations:	Three test concentrations, plus negative and solvent controls		*EPA recommends a minimum of 3 concentrations and a control, plus solvent control if appropriate.*
Are nominal concentrations adjusted for purity?	No		
Indicate the type of values presented for measured concentrations:	Mean-measured concentrations are presented		

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Limit of quantification (LOQ):	0.000015 mg a.i./L		*EPA recommends that for chemical test concentrations below the LOQ, analyses be conducted on the stock solutions.*
Level of detection (LOD):	Not reported		
Frequency of measurement:	Approximately 7 days		*It is recommended that test item concentration be measured in one tank at each treatment level at test initiation and every week thereafter.*
Number of replicates in control:	4		*EPA recommends 4 replicates.*
Number of replicates in solvent control (if applicable):	4		*EPA and OECD recommend the use of a concurrent solvent control when a solubilizing agent is used. EPA recommends 4 replicates.*
Number of replicates per test item treatment level:	4		*EPA recommends 4 replicates.*
Number of larvae per treatment at test initiation:	80		

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Was a solvent used?	Yes		
Solvent type (if applicable):	Dimethylformamide		
Maximum solvent concentration (if applicable):	0.02 mL/L		*EPA recommends that the solvent not exceed 0.02 ml/L*[3]*. OECD recommends that solvent have no effect on survival nor produce any other adverse effects and that concentration not be greater than 0.1 ml/L*[4]*.*
Was a positive control used?	No		
Positive control (if applicable):	Not applicable		
Positive control concentration(s) (if applicable):	Not applicable		
Photoperiod:	16 hrs light :		*EPA recommends photoperiod 12:12*

[3] Hutchinson TH, Shillabeer N, Winter MJ, Pickford DB (2006). Acute and chronic effects of carrier solvents in aquatic organisms: A critical review. Review. Aquatic Toxicology, 76, pp.69–92.

[4] OECD (2000). Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. Environmental Health and Safety Publications. Series on Testing and Assessment. No. 23. Paris, France.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
	8 hrs dark		*(light:dark).*
Light intensity at water's surface:	0.616 - 0.852 Klux; mean of 0.714 Klux		*EPA recommends light intensity 0.6 – 2 Klux (at water's surface).*
Additional details:	N/A		

Table 8: Summary of Treatment Concentrations in the Amphibian Metamorphosis Assay with Bifenthrin.

Treatment ID	Nominal Concentration (mg a.i./L)	TWA-Measured Concentration (mg a.i./L)	Mean CV (%)	Details or Remarks	Guideline Recommendations
Negative Control	0.00	<0.000015	N/A	N/A	*EPA and OECD recommend that test item concentrations be maintained at a coefficient of variation (CV) ≤20%.*
Solvent control	0.00	<0.000015	N/A	N/A	
Treatment 1	0.00003	0.000012	54.88	N/A	
Treatment 2	0.0001	0.000051	48.45	N/A	
Treatment 3	0.0003	0.00023	22.47	N/A	

Abbreviations: ^{CV} Coefficient of variation.

E. Observations

Biological Endpoints: Day 7: NF stage, wet weight, SVL, HLL, normalized HLL
Day 21: NF stage, wet weight, SVL, HLL, normalized HLL, thyroid histopathology
Daily- mortality and clinical signs

Were raw (individual) data provided? Yes

EPA recommends that observations of mortality and clinical signs occur daily, at a minimum; other observations are recommended as follows: NF developmental stage (days 7 and 21); any asynchronous development, indicated by tadpoles that cannot be assigned an NF stage (days 7 and 21); hind limb length (days 7 and 21); snout-vent length (days 7 and 21); body weight (test initiation, for optional size-based larval selection); and thyroid gland gross pathology and histopathology (day 21). Note the histopathology section of the test guideline also includes thyroid gross pathology observations.

II. RESULTS AND DISCUSSION

A. Results

After 7 days of exposure, mean survival was reportedly 100% in both the negative and solvent controls, and 100%, 96.3%, and 100% in the TWA-measured 0.000012, 0.000051 and 0.00023 mg a.i./L treatment groups, respectively. By test termination, mean survival was reportedly 96.7% in the negative control, 100% in the solvent control, and 100%, 94.9%, and 100% in the TWA-measured 0.000012, 0.000051, and 0.00023 mg a.i./L treatment groups, respectively. There were no treatment related effects on developmental stage, wet weight, snout to vent length or normalized limb length on Day 7. In both control groups, tadpoles were incidentally killed during siphoning and the study authors did not consider those deaths in survival calculations; actual survival data (including incidental deaths) are presented below in Table 9.

Table 9: Larval Mortality in *Xenopus laevis*.

Treatment (mg a.i./L) [TWA-measured]	Larval Mortality					
	Day 7[1]			Day 21		
	n	Mortality #	Mortality %	n	Mortality #	Mortality %
Negative Control	20	7	8.8	60	12	20
Solvent control	20	1	1.3	60	2	3
0.000012	20	0	0	60	0	0
0.000051	20	3	3.7	60	5	8
0.00023	20	0	0	60	0	0

Abbreviations: NA Not applicable.

[1] Sample size and cumulative mortality values at Day 7 prior to interim sacrifice.

By Day 7, the median developmental stage was 54 for the negative and solvent controls, and all treatment groups except for the TWA-measured concentration of 0.000051 mg a.i./L treatment group, which had a median developmental stage of 55 (Table 10). At test termination, the median development stage was 57 for the negative control and all treatment groups; the median developmental stage was 58 for the solvent control. No statistically significant or treatment-related effects on survival existed between the negative and solvent control groups (p >0.05); therefore the control data were pooled for comparison with the treatment groups. No statistically significant effects were found between any of the treatment groups and the pooled control (p > 0.05). Asynchronous development (stages 54 and 55) was observed in one tadpole at test termination in the TWA-measured concentration of 0.000051 mg a.i./L treatment group.

Table 10: Larval Development in *Xenopus laevis*. – Developmental Stage and Asynchronous Development.

Treatment (mg a.i./L) [TWA-measured]	Developmental Stage					
	Day 7			Day 21		
	n	Median Stage	# Asynchronous	n	Median Stage	# Asynchronous
Negative Control	20	54	0	48	57	0
Solvent control	20	54	0	58	58	0
0.000012	20	54	0	60	57	0
0.000051	20	55	0	55	57	1
0.00023	20	54	0	60	57	0

Abbreviations: [NA] Not applicable.

The Day 7 normalized HLL averaged 0.14 in the negative control and in the 0.00023 mg a.i./L treatment group (Table 11). The normalized HLL was 0.15 in the solvent control, the 0.000012 mg a.i./L treatment group and the 0.000051 µg a.i./L treatment group. The study author reported that the mean normalized HLL on Day 21 was 0.44 in the negative control, 0.48 in the solvent control, and 0.49, 0.48, and 0.39 in the TWA-measured 0.000012, 0.000051 and 0.00023 mg a.i./L treatment groups, respectively. No statistically significant or treatment-related effects on survival existed between the negative and solvent control groups ($p > 0.05$); therefore the control data were pooled for comparison with the treatment groups. No statistically significant effects were found between any of the treatment groups and the pooled control ($p > 0.05$).

Table 11: Larval Development in *Xenopus laevis* – Hind Limb Length.

Treatment (mg a.i./L) [TWA-measured]	Hind Limb Length (HLL)[1]							
	Day 7				Day 21			
	n	Mean (mm)	±SD	HLL: SVL[2]	n	Mean (mm)	±SD	HLL: SVL[2]
Negative Control	20	2.05	0.48	0.14	48	9.97	4.03	0.44
Solvent control	20	2.38	0.33	0.15	58	11.76	3.53	0.48
0.000012	20	2.34	0.29	0.15	60	11.35	4.19	0.49
0.000051	20	2.40	0.33	0.15	55	11.51	4.26	0.48
0.00023	20	2.07	0.46	0.14	60	9.12	4.08	0.39

Abbreviations: NA Not applicable. SD Standard deviation.

[1] Report the treatment mean measurements (mm) for hind limb length (HLL). Then report the normalized value (ratio of HLL:SVL) in the column following standard deviation (SD). The normalized value (HLL: SVL) reported in the table should be the treatment mean of individual ratios of HLL to SVL.

[2] Summary results for snout-vent length (SVL) are presented in the next table (Table 12).

Day 7 SVL averaged 14.7 mm in the negative control, 16.4 mm in the solvent control, and 15.6, 15.7, and 14.4 mm in the 0.000012, 0.000051 and 0.00023 mg a.i./L treatment groups, respectively (Table 12). By Day 21, SVL averaged 21.5 mm in the negative control, 23.4 in the solvent control, and 22.4, 22.3, and 21.9 mm in the 0.000012, 0.000051 and 0.00023 mg a.i./L treatment groups, respectively. No statistically significant differences were observed in any treatment group in comparison to the pooled control ($p > 0.05$) except in the highest dose treatment group on Day 7. This difference was significant using the Jonckeere-Terpstra trend test, but not according to Dunnett's test. Since the difference was no longer apparent by Day 21, it was not considered to be treatment-related.

Day 7 wet weight averaged 0.232 g in the negative control, 0.315 g in the solvent control and 0.279 g in the pooled control and 0.262, 0.275, and 0.227 g in the 0.000012, 0.000051 and 0.00023 mg a.i./L treatment groups, respectively (Table 12). No differences were detected following application of Dunnett's Multiple Comparison Test. Day 21 wet weight averaged 0.732 g in the negative control, 0.941 g in the solvent control and 0.851 g in the pooled control and 0.910, 0.886, and 0.836 g in the 0.000012, 0.000051 and 0.00023 mg a.i./L treatment groups, respectively. No statistically significant differences were observed in any treatment group in comparison to the pooled control ($p > 0.05$) except in the highest dose treatment group on Day 7. This difference was significant using the Jonckeere-Terpstra trend test, but not following application of Dunnett's test. Since this difference was no longer apparent by Day 21, it was not considered to be treatment-related.

Table 12: Larval Growth in *Xenopus laevis*

Treatment (mg a.i./L) [TWA-measured]	Snout-Vent Length (SVL)						Body Weight[1]					
	Day 7			Day 21			Day 7			Day 21		
	n	Mean (mm)	±SD	n	Mean (mm)	±SD	n	Mean (g)	±SD	n	Mean (g)	±SD
Negative Control	20	14.5	0.65	48	21.5	0.5	20	0.232	0.063	48	0.732	0.063
Solvent control	20	16.4	0.86	58	23.4	0.17	20	0.315	0.030	58	0.941	0.022
0.000012	20	15.6	0.27	60	22.4	0.42	20	0.262	0.009	60	0.910	0.043
0.000051	20	15.7	0.60	55	22.3	0.68	20	0.275	0.027	55	0.886	0.050
0.00023	20	14.4	0.81	60	21.9	0.51	20	0.227	0.032	60	0.836	0.034

Abbreviations: [NA] Not applicable. [ND] Not determined. [SD] Standard deviation.

[1] Also referred to as "wet weight" in the test guideline.

Mild to moderate thyroid gland hypertrophy, thyroid gland atrophy and follicular cell hypertrophy were observed in the negative control and solvent control groups. Mild thyroid gland hypertrophy and follicular cell hypertrophy

was observed in the 0.000012 mg a.i./L treatment group. Mild to moderate thyroid gland hypertrophy and atrophy and one incidence of moderate follicular hypertrophy were observed in the 0.000051 mg a.i./L treatment group. In the highest dose treatment group, mild and severe thyroid gland hypertrophy, mild to moderate thyroid gland atrophy and follicular cell hypertrophy, and one incidence of follicular cell hyperplasia were observed.

The decrease in follicle size was found to be statistically significant ($p=0.024$) in the high treatment group relative to the negative control. A significant correlation between gland area and snout-to-vent length in the pooled control (Pearson correlation coefficient = 0.74) was also found. A difference in normalized gland area between the pooled control and high treatment group was not significantly different using either the Jonckheere-Terpstra trend test or Dunnett's test.

Table 13: Gross Pathology and Histopathology of the Thyroid Gland in *Xenopus laevis.*

Treatment (mg a.i./L) [TWA-measured]	Diagnostic Observations[1]								
	Severity	Thyroid Gland Hypertrophy		Thyroid Gland Atrophy		Follicular Cell Hypertrophy		Follicular Cell Hyperplasia	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence
Negative Control	0	15	0	15	0	15	0	15	0
	1	15	1	15	4	15	2	15	0
	2	15	2	15	0	15	1	15	0
	3	15	0	15	0	15	0	15	0
Solvent control	0	20	0	20	0	20	0	20	0
	1	20	4	20	4	20	2	20	0
	2	20	1	20	1	20	2	20	0
	3	20	0	20	0	20	1	20	0
0.000012	0	20	0	20	0	20	0	20	0
	1	20	0	20	5	20	2	20	0
	2	20	0	20	1	20	0	20	0
	3	20	0	20	0	20	0	20	0
0.000051	0	20	0	20	0	20	0	20	0
	1	20	2	20	5	20	0	20	0
	2	20	1	20	1	20	1	20	0
	3	20	0	20	0	20	0	20	0
0.00023	0	20	0	20	0	20	0	20	0
	1	20	2	20	7	20	1	20	1
	2	20	0	20	2	20	2	20	0
	3	20	1	20	0	20	0	20	0

[1] Thyroid gland gross pathology and histopathology are graded 0 – 3 based on severity: 0=Not remarkable, 1=Mild, 2=Moderate, 3=Severe. See OECD No. 82 for reference.

Table 14: Additional Thyroid Gland Histopathology Observations in *Xenopus laevis*.

Treatment (mg a.i./L) [mean measured]	Additional Qualitative Observations[1]									
	Severity	Follicular Lumen Area (Increase)		Follicular Lumen Area (Decrease)		Follicular Cell Height (Increase)		Follicular Cell Height (Decrease)		Follicular Cell Shape	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence	n	Incidence
Negative Control	0	--	--	--	--	--	--	--	--	--	--
	1	--	--	--	--	--	--	--	--	--	--
	2	--	--	--	--	--	--	--	--	--	--
	3	--	--	--	--	--	--	--	--	--	--
Solvent control	0	--	--	--	--	--	--	--	--	--	--
	1	--	--	--	--	--	--	--	--	--	--
	2	--	--	--	--	--	--	--	--	--	--
	3	--	--	--	--	--	--	--	--	--	--
0.000012	0	--	--	--	--	--	--	--	--	--	--
	1	--	--	--	--	--	--	--	--	--	--
	2	--	--	--	--	--	--	--	--	--	--
	3	--	--	--	--	--	--	--	--	--	--
0.000051	0	--	--	--	--	--	--	--	--	--	--
	1	--	--	--	--	--	--	--	--	--	--

Treatment (mg a.i./L) [mean measured]	Additional Qualitative Observations[1]									
	Severity	Follicular Lumen Area (Increase)		Follicular Lumen Area (Decrease)		Follicular Cell Height (Increase)		Follicular Cell Height (Decrease)		Follicular Cell Shape	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence	n	Incidence
	2	--	--	--	--	--	--	--	--	--	--
	3	--	--	--	--	--	--	--	--	--	--
0.00023	0	--	--	--	--	--	--	--	--	--	--
	1	--	--	--	--	--	--	--	--	--	--
	2	--	--	--	--	--	--	--	--	--	--
	3	--	--	--	--	--	--	--	--	--	--
	1	--	--	--	--	--	--	--	--	--	--
	2	--	--	--	--	--	--	--	--	--	--
	3	--	--	--	--	--	--	--	--	--	--

[1] Thyroid histopathology is graded 0 – 3 based on severity: 0=Not remarkable, 1=Mild, 2=Moderate, 3=Severe. See OECD No. 82 for reference.

Tail curvature was present in the 42, 34, 47, 33 and 28% of the tadpoles in the negative control, solvent control, and in the 0.000012, 0.000051 and 0.00023 mg a.i./L treatment groups, respectively, at test termination. This was considered to be a dietary rather than treatment-related effect, as in feeding trials it was shown that feeding rates during acclimation contribute to the amount of curvature observed.

Table 15: Clinical Signs in *Xenopus laevis*.

Treatment (mg a.i./L) [measured]	Clinical Signs[1]		
	Type	n	Incidence
Negative Control	Tail curvature	48	32
	Small size		1
Solvent control	Tail curvature	58	34
	Small size		4
0.000012	Tail curvature	50	48
	Small size		1
0.000051	Tail curvature	55	36
	Small size		3
0.00023	Tail curvature	60	38
	Swollen abdomen		1

Treatment (mg a.i./L) [measured]	Clinical Signs[1]		
	Type	n	Incidence

[1] Define any abbreviations used to identify clinical signs (*i.e.*, sublethal effects, including behavioral effects) not specified elsewhere in the results. Examples include abnormal swimming behavior, lethargy, loss of equilibrium, curvature of the spine (*e.g.*, "bent tail"), other malformations, lesions, *etc.* Add rows as necessary. Note that asynchronous development (unable to stage) is reported previously in Table 10 and not here.

B. Study Author's Analysis and Conclusions

For each endpoint, data from the negative and solvent controls were compared using the appropriate statistical test. No differences were detected ($p > 0.05$) for any endpoint on Day 7 or 21 and all subsequent analyses were conducted by comparing treatment data to the pooled control. Unless otherwise noted, the unit of statistical analysis was the replicate test chamber. Most endpoints were analyzed using two complementary statistical approaches. If responses appeared to be monotonic a step-down Jonckheere-Terpstra trend test was used to evaluate trends in the ranks of replicate means to determine possible concentration responsive trends among the treatment groups. All endpoints except for survival, developmental stage, and histopathology severity scores also were analyzed by performing pair-wise comparisons using Dunnett's multiple comparison test or the Wilcoxon rank-sum test to determine which treatment groups differed statistically from the control group. Data for endpoints analyzed by Dunnett's test were evaluated for normality using Shapiro-Wilk's test and for homogeneity of variance using Levene's test ($\alpha = 0.01$). When the data did not satisfy the assumptions, a log transformation was applied to correct the data for non-normality or heterogeneous variances.

Survival, developmental stage, and histopathology severity scores were not amenable to the statistical methods used for analysis of other endpoints. In particular, the most suitable unit of statistical analysis for survival and histopathology severity scores was the individual animal. Therefore, survival was analyzed using the Fishers Exact test and the Jonckheere-Terpstra trend test, and histopathology severity scores of individuals were analyzed using the step-down Jonckheere-Terpstra trend tests only. In accordance with recommendations of OECD TG 231 and OPPTS 890.1100, analysis of metamorphic stage was performed using the multi-quantile analysis developed by T. Springer and J. Green, and using the step-down Jonckheere-Terpstra trend test on stage quantiles in the replicate test chambers. Statistical tests used to evaluate treatment effects were performed at a confidence level of $\alpha = 0.05$ with SAS software.

The study author found significant differences in the distribution of developmental stages between the pooled control and highest dose treatment groups that led to slight differences in normalized hind-limb

length. Following exclusion of all but Stage 57 tadpoles in the analysis, the 15% difference in normalized hind limb length was no longer present. Differences in gland atrophy in the higher treatment group were also found to not be statistically significant but within natural variability.

By Day 21, tail curvature was present in 42, 34, 47, 33 and 28% of the tadpoles in the negative control, solvent control, and in the 0.000012, 0.000051 and 0.00023 mg a.i./L treatment groups, respectively. In feeding trials it had been shown that feeding rates during acclimation contribute to the amount of curvature observed. Therefore, the tail curvature was not considered to be a thyroid-related effect, but rather a dietary effect.

C. Reviewer's Analysis and Conclusions

Statistical Methods Negative and solvent control data for each endpoint were compared using an Equal Variance Two-Sample t-test. Solvent control data were significantly greater ($p<0.05$) for Day 7 wet weight, SVL, and developmental stage, and for Day 21 wet weight, SVL and HLL. Subsequent comparisons to treated groups were made using only the negative control and, unlike the study authors, the reviewer included data for all living individuals (NF<60) from replicate A of the negative control in the statistical analysis of Day 21 endpoints.

No monotonic dose-dependent trends were identified for any endpoint. All endpoints were tested for normality using Shapiro-Wilks test (α = 0.01) and for homogeneity of variances using either Levene's or Bartlett's test (α = 0.01). All endpoints met the assumptions of parametric statistics and were analyzed using Dunnett's Multiple Comparison test. These analyses were conducted using CETIS 1.8.7.7 and backend settings implemented by EFED on 5/29/13.

Late-stage tadpoles were found in both control groups and all treatment groups; however, in no case did the occurrence of late-stage tadpoles warrant a separate analysis (i.e., no more than 20% late stage individuals in any test level). In the negative control, solvent control, 0.000012, 0.000051, and 0.00023 mg a.i./L groups there were a total of 3, 3, 2, 4, and 2 NF$\geq$61 individuals, respectively, and NF stage of these individuals ranged from 61 to 63 with no apparent pattern in their occurrence. There was one

incidence of asynchronous development observed in the middle dose treatment group, but this isolated incident was not considered to be treatment-related. Unless otherwise indicated, effects were considered statistically significant at $p < 0.05$.

Conclusions:

Day 7 HLL, SVL, and wet body weight were significantly increased in the 0.000051 mg a.i./L treatment group relative to the negative control (Dunnett's, $p<0.05$); there were no other Day 7 parameters affected by treatment. Day 21 median developmental stage was significantly delayed in the 0.00023 mg a.i./L treatment group relative to the negative control (median NF=58 vs. 57, respectively; Dunnett's, $p<0.05$). In terms of growth parameters, Day 21 SVL was significantly increased at the 0.000012 mg a.i./L treatment level, relative to the negative control ($p<0.05$; Dunnett's). Day 21 wet body weight was significantly greater in all treatment groups, relative to the negative control ($p<0.05$, Dunnett's). For every endpoint except Day 21 development stage, the solvent control response was promoted (and in most cases significantly so, $p<0.05$) relative to the negative control response, suggesting that there may have been solvent interference in this study (promoted response due to solvent may have masked potential adverse effects of the test material; in most cases, the direction of the response relative to the negative control was reversed to a reduction at the high dose level). Based on the significant effects on developmental stage, the test material may interact with the HPT of *Xenopus laevis* tadpoles under the conditions and using nominal concentrations employed in the current test. These results should be interpreted with caution because of the implication of solvent interference, and due to the high incidence of negative control mortality which compromised a replicate from this group for many Day 21 endpoints, reducing the statistical power of comparisons made to this reference group.

Table 16: Developmental and Thyroid Gross Pathology/Histopathology Endpoints[1,2] in the AMA with Bifenthrin.

TWA concentrations (mg a.i./L) [measured]	NF Developmental Stage				Hind Limb Length[3]				Asynchronous Development				Thyroid Gross and Histopathology
	Day 7		Day 21		Day 7		Day 21		Day 7		Day 21		Day 21
	Median	p	Median	p	% Diff.	p	% Diff.	p	%	p	%	p	Treatment-Related Effects? (Yes/No)
Negative Control	53.5	NA	58	NA	0	NA	0	NA	NA	NA	0	NA	No
Solvent Control	54	**0.0498**	58	0.3559	3.57	0.3559	14.20	0.0823	0	NA	0	NA	No
0.000012	54	0.3221	57.5	0.6861	7.14	0.3482	15.38	0.1073	0	NA	0	NA	Yes
0.000051	54	0.0937	58	0.6861	8.93	0.1993	12.43	0.2171	0	NA	2	NA	Yes
0.00023	54	0.7818	57	**0.0422**	0	1.000	-7.1	0.6144	0	NA	0	NA	Yes
Statistical Analysis	Dunnett's				Dunnett's				NA				No

Abbreviations: Diff. Difference. NA Not applicable.

[1] Unless otherwise indicated, effects are reported based on comparison to the clean water control. Conclusions regarding histopathology may be heavily weighted by the expert opinion of a board-certified pathologist.

[2] Unless otherwise specified, effects are considered statistically significant at [$p<0.05$].

[3] Hind-limb length is normalized to snout-vent length (SVL).

Table 17: Growth Endpoints[1,2] in the AMA with Bifenthrin.

TWA concentrations (mg a.i../L) [measured]	Snout-Vent Length				Body Weight			
	Day 7		Day 21		Day 7		Day 21	
	% Diff.	p	% Diff.	p	% Diff.	p	% Diff.	p
Negative Control	0	NA	0	NA	0	NA	0	NA
Solvent Control	13.31	**0.0119**	10.64	**0.0016**	43.15	**0.0058**	35.58	**0.0019**
0.000012	7.59	0.0730	5.79	**0.0437**	19.11	0.1232	31.15	**0.0006**
0.000051	8.28	**0.0492**	5.32	0.0652	25.26	**0.0366**	27.62	**0.0016**
0.00023	-0.86	0.9844	3.31	0.3100	3.41	0.9616	20.45	**0.0134**
Statistical Analysis	Dunnett's				Dunnett's			

Abbreviations: [Diff.] Difference. [NA] Not applicable.

[1] Unless otherwise indicated, effects are reported based on comparison to the negative (clean water) control.

[2] Unless otherwise specified, effects are considered statistically significant at [$p<0.05$].

E. Study Deficiencies

There were several critical deficiencies in this study:

1. The %CV of measured concentrations exceeded 20% over the 21 day study (CV ranged from 22.5 – 54.9%). As a result, the reviewer calculated and expressed toxicity values using TWA concentrations.
2. Mortality in negative control replicate A (45%) and consequently, the negative control group as a whole (20%), exceeded the acceptable mortality rate (<10%). This reduced sample size for Day 21 endpoints compromised the statistical power with which to make comparisons to the treated levels at this time period.
3. Additionally, there was a promoted response evident for virtually every endpoint in the solvent

control group, relative to the negative control. This suggests that there may have been solvent interference in this study and potential masking of adverse test material effects.

The remaining performance and validity criteria were satisfied.

F. Reviewer's Comments

The reviewer's results were determined by comparing treatment data to the negative control, whereas the study authors compared treatment data to the solvent or pooled control. Therefore, the reviewer's results are reported in the Executive Summary and Conclusions sections of this DER.

The reviewer calculated the time-weighted average (TWA) concentrations using the following equation:

$$C_{TWA} = \frac{\left(\frac{C_1 + C_0}{2}\right)(t_1 - t_0) + \left(\frac{C_2 + C_1}{2}\right)(t_2 - t_1) + \left(\frac{C_{n-1} + C_2}{2}\right)(t_{n-1} - t_2) + \left(\frac{C_n + C_{n-1}}{2}\right)(t_n - t_{n-1})}{t_n}$$

where:

C TWA is the time-weighted average concentration,

C j is the concentration measured at time interval j (j = 0, 1, 2,...n)

t j is the number of hours (or days or weeks, units used just need to be consistent in the equation) of the test at time interval j

(e.g., t 0 = 0 hours (test initiation), t 1 =24 hours, t 2 =96 hours)

The feeding rates for tadpoles during the definitive study increased from 15 mg/larvae/day at test initiation to 40 mg/larvae/day by the last week of the test, which was not consistent with the recommended feeding rate of 30 mg/larva/day at test initiation to 80 mg/larva/day in the last week of the test.

III. REFERENCES

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